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Podophyllotoxin directly binds a hinge domain in E2 of HPV and inhibits an E2/E7 interaction in vitro

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Abstract—Podophyllotoxin (PT), a strong cytotoxic agent from berberidaceae, has been known to inhibit tubulin polymerization. Although PT has been used for developing anticancer drugs as one of seed compounds, clinical treatment by itself has been unsuccessful because of the side effects, except one example in the treatments of warts. In this study, we screened peptides binding to PT with T7 phage display clonings in order to obtain more information about molecular mechanism of the action. A selected phage clone has a specific amino acid sequence to be SVPSRRRPDGRTHRSSRG. A homology search by protein database BLAST showed that this sequence had a similarity to a hinge domain (HD) of E2 protein in human papillomavirus (HPV) type 1a which is known to cause plantar warts. Surface plasmon resonance (SPR) analysis showed that PT bound to a recombinant HPV 1a E2 protein giving a K_D = 24.1 μM which has compared with those of other domains of E2 protein. Also we demonstrated whether PT inhibited HD interaction or not. E7 protein of HPV has been known to be an oncoprotein and was reported to interact with HD of E2 protein. We demonstrated that an E2/E7 interaction was inhibited by the addition of PT in this report. And we showed the bindings of PT to other types of HPV. Our results suggest that PT is potential as a tool for clarifying the molecular mechanism of HPV.

1. Introduction

Podophyllotoxin (PT), which is a lignan extracted from roots of *Podophyllum peltatum,* has a strong cytotoxicity in several cells (Fig. 1a).[1,2] Etoposide (EP), one of semi-synthetic derivatives of PT, has been widely used as an anti-cancer drug and has given good clinical results against several types of neoplasm (Fig. 1b).[3] Although a cytotoxic effect of EP can be based on the inhibition of DNA topoisomerase II, PT acts as an inhibitor of tubulin polymerization.[4,5] Podophyllin, which contains PT as a constituent, is a resin from the roots and rhizomes of *P. peltatum*, and has been widely used as a treatment for genital warts (known as condyloma acuminate) caused by human papillomavirus (HPV).[6] The mechanism responsible for the clinical effects could be

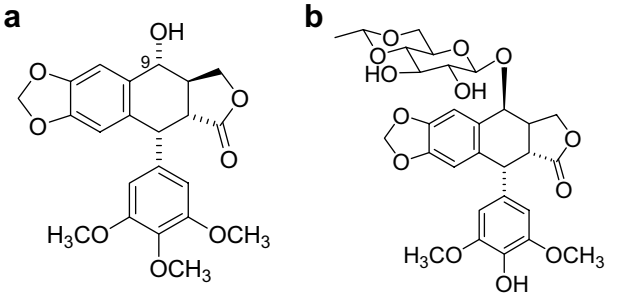

Figure 1. Structures of podophyllotoxin and its derivative. (a) Podophyllotoxin. (b) Podophyllotoxin derivative to inhibit topoisomerase II, etoposide.

Keywords: HPV; Podophyllotoxin; E2; Phage display; SPR.
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owed that PT induced necrosis in host cells and resulted to disrupt viral replications.[7] However, direct influence of PT on HPV itself is still ambiguous.

Human papillomavirus is a family over 100 types of viruses, some of which cause diseases such as hand warts, plantar warts, genital warts, and several cancers.[8,31] HPVs are maintained as episomal circular double-strand DNA in infected cell and are replicated by the virus proteins E1 and E2.[9,29] The E2 protein, which is one of the functional proteins of HPV, works as a transcriptional activator and repressor for virus. After infection to host cells, the complex of E2 and E1 proteins down-regulates other viral proteins on its genome and starts viral proliferation. HPV genome also contains three oncogenes, E5, E6, and E7 and two structural proteins, L1 and L2. In some types of HPV to be known as a responsible virus for developing cervical cancer, E6 and E7 proteins regulated by E2 protein interact with p53 and Rb proteins and turn host cells to cancer.[10,11] E2 protein composes three domains, activator domain (AD), HD, and DNA-binding domain (DBD). AD is an N-terminal domain of E2 protein, and then activates transcription and replication of the site of E2 protein interacting with E1 protein is conserved on AD.[30,32] A C-terminal domain, DBD, binds to specific sequence $ACCN_6GGT$ and formed DNA–protein complex. The structure of DBD/DNA complex has been reported, and DBD is known to induce apoptotic cell death and bind to p53.[12,13,38] Both domains, AD and DBD, are found in many types of HPV, and each function is well studied.[12,33] On the contrary to them, HD is constituted with flexible amino acids sequence and the homology among HPV types is lower than those of other two domains, and its function is still unknown. In a recent study, however, direct interactions of HDs in each HPV 11 and 16 E2 protein with E7 protein was observed in vivo and in vitro by Gammoh et al.[14] And Zou et al. demonstrated that HD contained critical determinates for nuclear localization and nuclear matrix association.[15] Lai et al. showed that E2 protein of HPV 5 interacted to SR protein with RS rich region in HD and assisted pre-mRNA splicing.[16] The function of HD was thought to work as a linker between two functional domains, but the significance of HD function has been recently recognized.

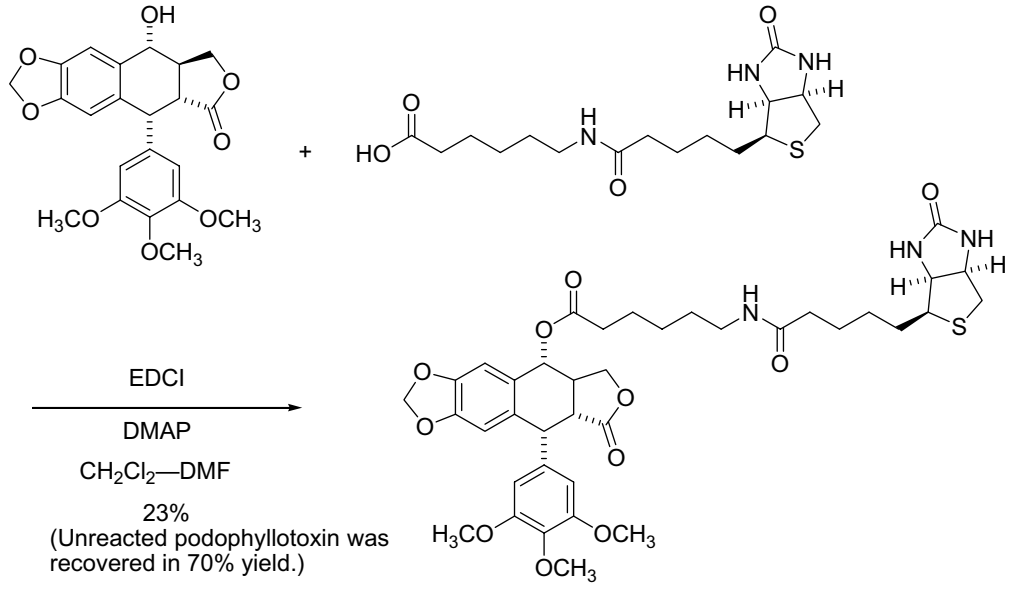
In order to obtain broad information on the binding molecules of PT, we focused on T7 phage display.[17,34] T7 phage particles can exhibit a huge number of proteins and peptides, which are derived from foreign DNA insert, on coat proteins as fusion proteins. After affinity selections of a phage library to a target molecule, peptides or proteins that bind to the molecule can be easily elucidated by sequencing insert DNA in the affinity phages. This method allows a rapid selection and an identification of binding peptides or protein to the target molecules of interest. Recently, several binding proteins or binding sites of small molecules have been determined by using the phage display technology.[18–20]

We report here in a T7 phage display screening of PT-binding molecules and possibilities of direct interaction between PT and papillomavirus proteins.

2. Result

2.1. Synthesis of biotynilated podophyllotoxin and assay for cytotoxicity

Studies on structure–activity relationships of PT by López-Pérez et al. established that the acylation of the hydroxyl group at C-9 did not influence on the cytotoxicity.[22] Therefore we decided to connect a biotin moiety to the hydroxyl group C-9 in PT. PT was coupled with biotinylaminocaproic acid to furnish biotinylated PT (Bio-PT) in 23% yield (70% yield based on the recovered PT) (Scheme 1).



Scheme 1. Synthesis of a biotinylated podophyllotoxin (Bio-PT).

We determined the cytotoxicity against murine leukemia cell line L1210. Bio-PT had cytotoxicity against L1210 cells with IC_{50} values of 400 nM, which was about 60-fold less potent than that of PT (7 nM). Nevertheless, Bio-PT still kept the cytotoxicity and would work as a tool for identifying the binding molecules of PT.

2.2. Screening of peptides selectively binding to PT by phage display and homology search

Peptides specifically binding to PT were screened from T7 phage library constructed with *Drosophilae* random cDNA by using Bio-PT which immobilized streptavidin-coated wells.[20] For screening, a suspension of 1.0×10^8pfu/well of phage library 200 μL was applied onto each well. The wells were washed with 200 μL of 100 mM Tris–HCl (pH 8.0), followed by the wash buffer (3% Tween 20, 2 M urea, 3 M NaCl, 100 mM Tris–HCl, pH 8.0) to eliminate unbound and non-specifically bound phage particles. The remainings on the wells were recovered by infection of the log phase *E. coli* (200 μL) applied on the wells. After four rounds of biopanning according to this method, the DNA sequences of randomly selected 96 clones were analyzed. Thus, we found that single clone was highly concentrated (Fig. 2a). The PT-binding clone particles conserved a specific cDNA sequence to encode 18 amino acids (SVPSRRRPDG RTHRSSRG) on the surface.

In order to check the binding specificity of the peptide against PT, the clone was amplified and the resulting single clone was applied for the binding assay.[23] The phage particle with no inserted cDNA fragment (NoIns phage) was used for the same binding assay to be a negative control. We compared the recovery titers of each clone from PT immobilized wells (PT(+) wells) and non-immobilized wells (PT(−) wells) (Fig. 2b). The titers of the PT-binding clone derived from PT(+) wells were 5-fold higher than those from PT(−) wells. On the other hand, there is no difference between PT(+) wells and PT(−) wells in the recovered titer of the NoIns phage clone. These results indicated that the PT-binding clone was effectively concentrated in the biopanning to be due to the specificity.

A homology search on BLAST demonstrated that the amino acid sequence displayed on the PT-binding clone was similar to that of the hinge domain (HD) in the E2 protein of human papillomavirus (HPV) type 1a (Fig. 2c).[13–16] We focused on the fact that PT were used as a treatment for genital warts caused by HPV, and we then decided to examine the effect of PT on HPV E2.

2.3. Kinetic analysis of the binding between PT and HPV 1a E2 protein by SPR

At first, we investigated the interaction between a recombinant HPV E2 protein and PT by surface plasmon resonance (SPR) analysis. The interaction between PT and recombinant full length HPV 1a E2 protein was analyzed by SPR biosensor instrument, Biacore3000 (GE Healthcare). The several concentrations of PT were employed to determine the binding to the conjugated recombinant proteins on CM5 sensor chip (Fig. 3a). The binding between PT and full length 1a E2 was analyzed with the BIAEVALUATION 4.1 software and the dissociation constant K_D(M) was determined by using general fitting analysis. The K_D value of PT binding to full length 1a E2 was determined to be 24.1 μM. And then, we also observed the interaction and determined the K_D value of EP binding to full length 1a E2 (Fig. 3b). The binding activity of EP to HPV 1a E2 protein was 10-fold weaker than that of PT (EP: K_D = 210 μM).

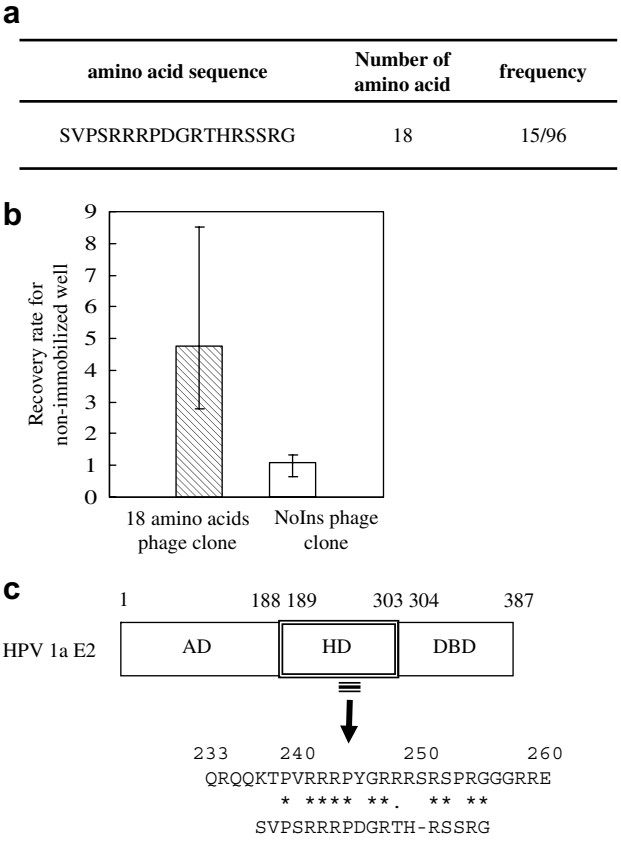
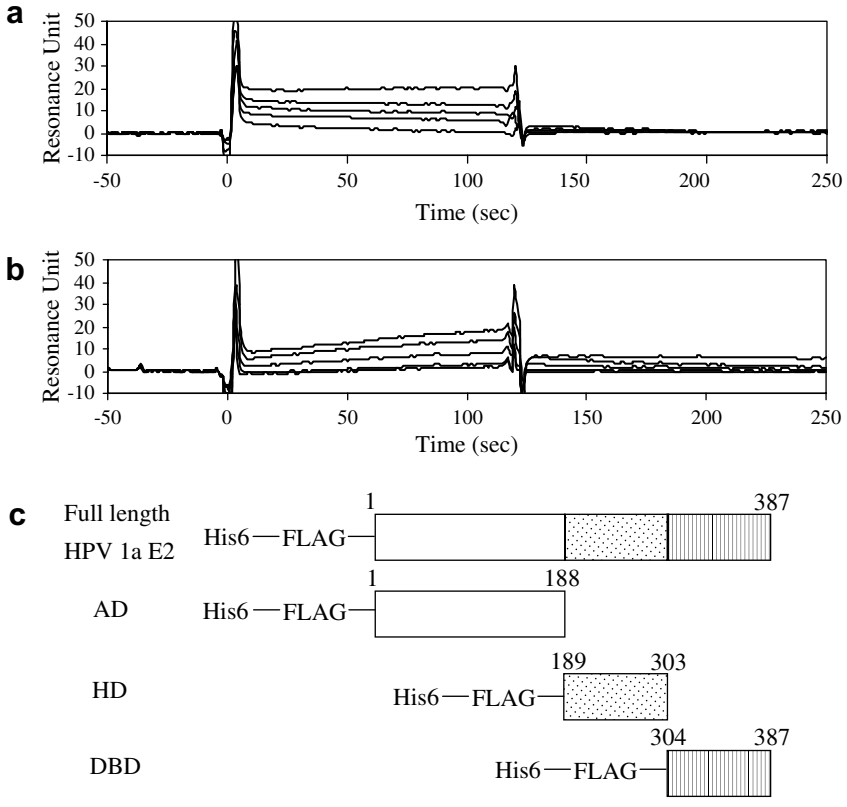


Figure 2. Result of screening with T7 phage particles constructed from a random cDNA library of *D. melanogaster*. (a) Sequence result obtained from concentrated phage particles after four rounds of biopanning. DNA sequence analysis of 96 phage plaques showed 18 amino acids clone concentrated up to 15 clones, 15.6%. (b) Single clone biopanning demonstrated 18 amino acids clone had specificity of binding to Bio-PT immobilized to streptavidin-coated wells compared with non-insert (NoIns) phage clone. Recovery rate was calculated by (titer of PT-immobilized well/titer of non-immobilized well). (c) Homology of 18 amino acids clone to HPV 1a E2 HD estimated by BLAST.

In order to confirm the binding domain of PT in HPV 1aE2 protein, we next expressed and purified recombinant each domain, AD, HD, and DBD. Then the bindings between PT and each domain were analyzed by the Biacore apparatus (Fig. 3c and d). The K_D value obtained from HD, which has a similar sequence to 18 amino acid sequence displayed on PT-binding phage clone, was estimated to be 1.05 μM. However, the K_D values obtained from AD and DBD, which have no similarity to 18 amino acid sequence, showed that both



d

Domain of HPV 1a E2	K_D (μM)
full length	24.1 ± 3.1
AD	>400
HD	1.05 ± 0.44
DBD	>400

Figure 3. SPR instrument analysis of interaction between PT and E2 protein. (a) and (b) SPR (Biacore 3000, GE healthcare) sensorgram of compounds (a, PT; b, EP) with immobilized full length 1a E2 protein on CM5 chip. The concentrations of compounds were 100, 50, 25, 12.5, 6.25 μM from up to bottom. Kinetic values were analyzed by BIAEVALUATION 4.1 (GE healthcare) and were determined (PT, K_D = 24.1 μM; EP, K_D = 210 μM). (c) Construct for recombinant proteins of HPV 1a E2 each domain. To be solved easily, FLAG tag was inserted instead of T7 tag. After infection by *E. coli*, the expressed proteins were extracted and purified on a FPLC system. (d) Kinetics values of PT and HPV 1a E2 each domain. SPR analysis was performed at flow late 20 μL/min and at 25 °C with running buffer. Each recombinant protein was immobilized on CM5 chip with amine coupling reaction, and sensorgrams were analyzed by BIAEVALUATION 4.1.

domains did not bind to PT (K_D > 400 μM). When we attempted electro mobility shift assay (EMSA) with recombinant E2 protein, PT had no inhibition between the E2–DNA interaction (data not shown).[24] Thus the results demonstrated here suggested that PT specifically binds to the HD in HPV 1a E2 protein, implying that PT binds to the amino acid sequence, which is similar to that selected by the phage screening.

2.4. Direct protein–protein interaction between E2 and E7 of HPV 1a observed by SPR

Gammoh et al. reported that E2 proteins of HPV 11 and 16 interact with each E7 protein directly by HD in vivo and in vitro.[14] We obtained the interacting from E2 protein and E7 protein of HPV 1a by SPR. We prepared the recombinant E2 and E7 proteins of HPV 1a to be His tag fusion proteins respectively, and analyzed the interaction between them. The E7 protein was immobilized on a CM5 sensor chip, and the E2 protein was applied as an analyte. The binding of E2 protein on E7 protein was observed at various concentrations (Fig. 4a). The K_D value of 0.73 μM was obtained by separate fitting of the sensorgrams to a 1:1 Langmuir model. The result implied that the interaction between HPV 1a E2 and E7 proteins was performed in vitro by the SPR experiments.

2.4.1. Inhibition of the E2/E7 interaction by PT. We demonstrated that PT bound to HD of HPV the E2 and that 1a E2 protein interacted with E7 protein obtained from the SPR experiments as described above. We next surveyed whether PT inhibit this interaction or not. We

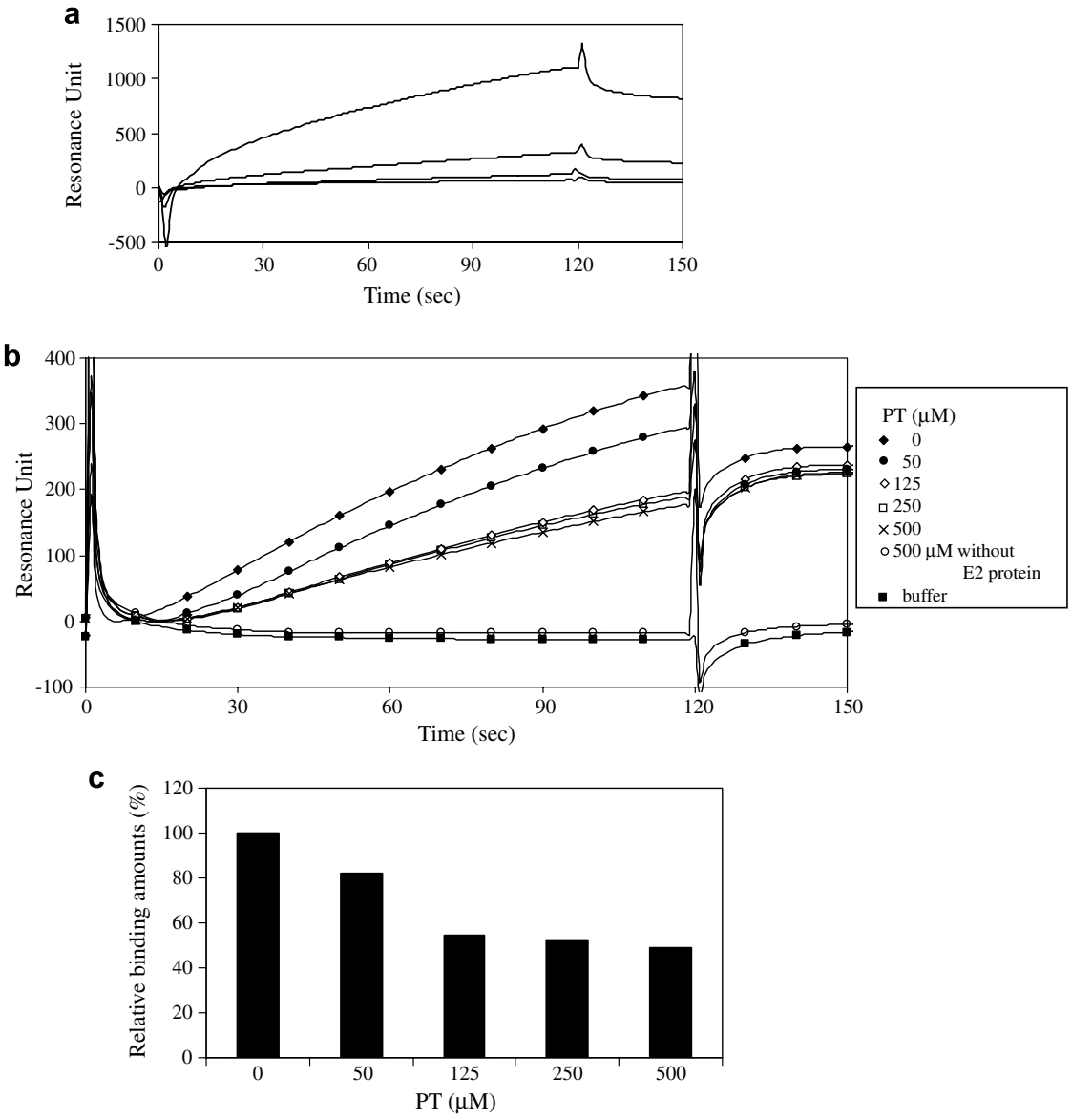


Figure 4. HPV 1a E2/E7 protein–protein interaction was determined with SPR and inhibited by PT. (a) Sensorgram of E2 protein with immobilized E7 protein. The concentrations of E2 protein were 1.0, 0.5, 0.25, 0.125 μM from up to bottom. And then dissociation constant (K_D) was determined to be 0.73 μM from this sensorgram with BIAEVALUATION 4.1. (b) Inhibition analysis of E2/E7 interaction by PT with SPR. Tested solutions of E2 protein were adjusted to 0.1 μM concentration contained with several concentration of PT in 5% DMSO. Analysis was performed at flow rate 30 μL/min and at 25 °C. Buffer and 500 μM PT without E2 protein were showed no effect on sensorgram. (c) Comparison with bound E2 protein amounts at 115 s after injection. Resonance unit of control (PT 0 μM) was regarded as 100% and the amounts of bound E2 protein with immobilized E7 protein were reduced by the presence of PT.

monitored the amounts of E2 protein bound to immobilized E7 protein in the presence or absence of PT by SPR.[35] After a solution of 0.1 μM E2 protein was premixed with several concentrations of PT in 5% DMSO, the resulting solution of the mixture was employed for analysis of the binding to E7 protein. The bound amounts of E2 protein was determined from the response units at 115 s (Fig. 4b and c). When the bound amounts of E2 protein on E7 protein in the absence of PT was regarded as 100%, the binding amounts of E2 protein decreased in the presence of PT. Finally, the rate of binding by E2 protein to E7 protein at concentration of 500 μM PT reached up to 49.1%. We also tested whether PT bound to E7 protein, however, PT did not bind or weakly bound to E7 protein (data not shown). These results suggested that PT bound to directly E2 protein and inhibited the protein–protein interaction between E2 and E7 in vitro.

2.4.2. Comparison the abilities to inhibit E2/E7 interaction between PT and EP. The K_D value in the EP/E2 interaction was 10-fold weaker than that in the PT/E2 interaction (Fig. 3a and b). And the inhibition of HPV 1a E2/E7 interaction by PT was as above mentioned

(Fig. 4b). Then, we also checked the inhibition by EP. The amounts of E2 protein bound to E7 protein was decreased to 82.0% and 60.6% by the addition of 250 μM of EP and PT, respectively (Fig. 5a and b). These results suggested that the binding abilities of the proteins can be responsible to the difference of inhibition activities.

2.5. Kinetic analysis of the binding between PT and other HPV E2 proteins, HPV 11 and 16, by SPR

Having found that PT bound to E2 proteins of HPV 1a, we assessed whether PT bound other E2 proteins derived from other HPV types. Amino acid sequences of AD and DBD in HPV E2 have been widely known to be highly conserved between many types.[13,25] On the other hand, HD has been known to be scarcely conserved between many HPV types. And the homologies between 18 amino acids and amino acid sequences of HD in other types such as high risk type HPV 16 and low risk type HPV 11 has been checked (Fig. 6a). The similarities in both HD to 18 amino acid sequences were lower than that in HPV 1a. However, the K_D values for the binding between PT and recombinant HPV 11 and 16 E2 proteins which expressed in *Escherichia coli* were not so much difference to that of HPV 1a (Fig. 6b and c). These results would indicate that PT bound to other types of E2 protein in spite of the lower homologies between E2 proteins and amino acid sequence obtained from phage display screening in this text.

3. Discussion

Podophyllotoxin (PT) is a natural lignan that has strong cytotoxicity against several cancer lines. However, the clinical use of PT in the treatment of cancer has been limited by severe toxic side effects.[4] The cytotoxicity of PT is considered to be due to its inhibitory effect of tubulin polymerization. On the other hand, etoposide (EP), a semisynthetic derivative of PT, is clinically used as an anticancer drug. EP induces topoisomerase II-mediated DNA cleavage, however, it has no effect on the tubulin assembly. We have been interested in the difference of biological properties between PT and EP.

In order to screen binding molecules of PT, we prepared the biotinylated PT derivative (Bio-PT) (Scheme 1). Biotin moiety was substituted on a hydroxyl group at C-9 position in PT by a condensation reaction. It has been reported that the hydroxyl group at C-9 position in PT was essential for the inhibitory activity of tubulin polymerization.[27] However, some 9-*O*-acyl derivatives still have cytotoxicity, while they lost the inhibitory activity of tubulin polymerization.[4,26–28] Actually, our Bio-PT showed cytotoxicity against L1210 cell line with IC_{50} value of 400 nM. Although the cytotoxicity of Bio-PT was less potent than that of PT (IC_{50} value of 7 nM), it was still strong. These results strongly suggested the presence of other target molecules of PT. We applied a T7 phage display method to screen for PT-binding mol-

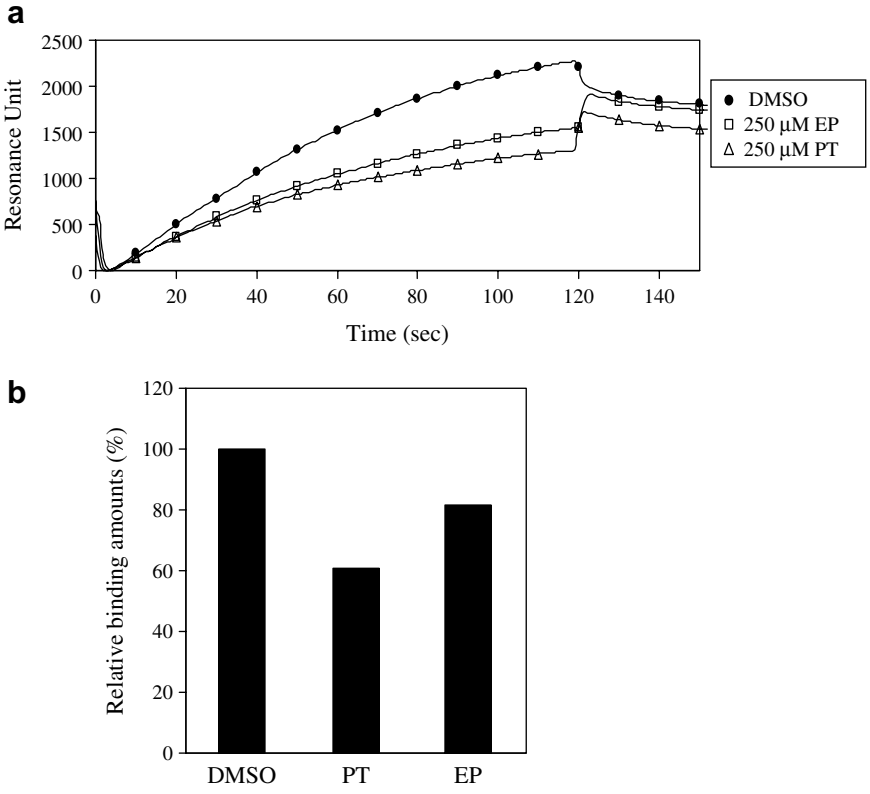


Figure 5. Comparison with PT and EP at inhibition of E2/E7 interaction. The concentration of E2 was adjusted to 0.5 μM. (a) EP less inhibited E2/E7 interaction than PT. (b) At 115 s after injection, a solution of 250 μM PT inhibited E2 protein binding activity about 60% compared with a solution of 5% DMSO, but a solution of 250 μM EP inhibited about 80%.

a

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          230       240       250       260       270
HPV 11 SACTTEDGVSAPPRKRARGPSTNNTLCVANIRSVDSTINNIVTDNY
                *.*.*:*. *            *:  *:
       ---------SVPSRRRPDG-----------RTHRSSRG-------

      202     210       220       230       240       250       270
HPV 16 SNEVSSPEIIRQHLANHPAATHTKAVALGTEETQTTIQRPRSEPDTGNPCHTTKLLHRDSVDS
           * *                                .* .**           **.*
       ----SVP--------------------------------SRRRPDG--------RTHRSSRG-
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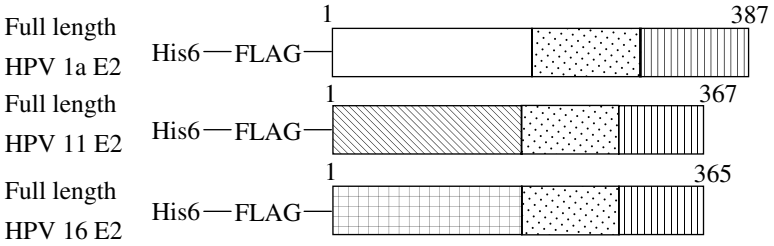
b



c

HPV type	K_D (μM)
1a	24.1 ± 3.1
11	20.1 ± 0.7
16	70.5 ± 8.5

Figure 6. Analysis for interaction between PT and HPV 11 and 16 E2 proteins. (a) Alignment between 18 amino acids sequence and hinge domain of HPV 11 and 16 E2 proteins obtained by CLUSTALW. The results of alignments showed that 18 amino acids sequence was similar to the part of hinge domain in HPV 11 E2 (S235-G243). But this peptide was not matched enough to HPV 16 E2 on the primary structure. (b) Construction of recombinant HPV E2s. These proteins were expressed and purified same as HPV 1a E2 protein. (c) Comparison among K_D values by Biacore 3000 at three types HPV E2 proteins with PT. These results were obtained by same methods of Figure 3a and b.

ecules. After screening, we obtained a phage clone, which conserved 18 amino acids (SVPSRRRPD GRTHRSSRG), and we proved that this clone bound PT by binding assay. Although the NoIns clone had no affinity in the PT-immobilized wells, the selected clone showed affinity in the PT-immobilized wells 5-fold greater than that in non-immobilized wells.

One advantage to display the drug-binding amino acid sequences on phage particles is that the technique can also identify a binding site within the binding protein.[18,23] Inversely, the candidates for the binding proteins can be estimated by analyzing the peptide sequences selected by phage display screening. Thus, we analyzed the selected sequence (SVPSRRRPDGR THRSSRG) to obtain clues for PT-binding proteins. A homology search of the selected sequence by using BLAST programs gave several candidates for PT-binding proteins. Among them, our tension has focused on a HPV 1a E2 protein. The selected peptide was similar to a part of the hinge domain of HPV 1a E2 protein. HPV 1a has been known to cause plantar warts, and PT has been clinically used for the treatment of plantar and genital warts caused by HPVs.[2,36] However, the interactions between PT and proteins of HPV have never been studied before.

The E2 protein plays an important role as a major regulator of both viral transcription and DNA replication. The E2 protein has three domains, a transcriptional activator domain (AD) in the amino-terminal domain, a hinge domain (HD) of the intermediate region between two functional domains, and a DNA-binding domain (DBD) in the carboxyl-terminal domain. E2 interacted at its HD region with several cellular proteins, such as E7 protein, nuclear matrix, and pre-mRNA splicing factor.

The binding between PT and HPV 1a E2 protein was confirmed by a SPR experiment (Fig. 3). PT binds HPV 1a E2 protein with a dissociation constant (K_D) of 24.1 μM. PT is known as a tubulin polymerization inhibitor, and the K_D value for PT binding to tubulin was reported to be 0.6–0.7 μM.[37] The K_D value for tubulin is 40-fold stronger than the value for full length HPV 1a E2 protein, but these values were determined with purified proteins in vitro, and clarifying the relationship between K_D values and efficacy of PT depends on the further studies. We also found that PT selectively interacted with the hinge domain of HPV 1a E2 protein, while it did not show any affinity with other domains (AD and DBD) (Fig. 2 and 4c). These results indicated that PT selectivity bound to the hinge domain of HPV 1a E2 protein, and suggested that PT binds to the amino acid sequence, which was similar to that selected by phage display screening. Interestingly, EP weakly interacted with HPV 1aE2 protein (Fig. 3b). Thus, the trimethoxyphenyl moiety at E ring or the stereochemistry at C-9 in PT will be important for the interaction between PT and HPV 1a E2 protein.

Gammoh et al. reported that E2 protein of HPV 11 or 16 directly bound with the corresponding E7 protein at HD in vitro and in vivo.[14] Thus, we next investigated the effect of PT on the interaction between HPV 1a E2 protein and E7 protein. We confirmed that HPV 1a E2 protein bound with HPV 1a E7 protein in vitro by SPR (Fig. 4a). The obtained K_D value for E2 protein with E7 protein was 0.73 μM. The interaction between E2 protein and E7 protein was inhibited by addition of PT (Fig. 4b and c). The relative binding ratio decreased about half when 500 μM of PT was added, indicating that PT apparently inhibited the interaction between E2 protein and E7 protein. We also found that the inhibitory effect of EP was much weaker than that of PT (Fig. 5).

We identified that PT inhibited the interaction between HPV 1a E2 protein and E7 protein. Although the inhibitory effect is weak, this is the first example of E2 protein inhibitor which inhibits E2/E7 protein–protein interaction. Known E2 protein inhibitors target the interactions between E2 protein and DNA, or between E2 protein and E1 protein.[39,40] Since our results showed that PT bound to the HD of HPV 1a E2, and the HD was reported to interact with several transcription factors, PT might inhibit the interaction of E2 protein and other proteins. The interaction between E2 protein and other cellular factor will be studies in due course. The molecular mechanism of HPV infection, proliferation, and cancer formation has not been clarified yet. The modification and structure optimization of PT as an E2 protein inhibitor will develop powerful tools to reveal the function of E2 protein as well as the virus life cycle.

PT has been used as a treatment for genital warts including condyloma acuminate. HPV 1a is known to cause plantar warts, while genital warts are caused by HPV 11 mainly.[7] Our results suggest that PT influences to E2 function HPV 1a by binding. Furthermore, since we aimed to assess the relationship between the potency of PT and HPV 11, at first, we searched a homology between HPV 11 E2 protein and 18 amino acids, which we obtained by screening (Fig. 6a). When we observed the interaction between HPV 11 E2 protein and PT, we tested about E2 protein of HPV 16 in order to compare to HPV 1a and 11. HPV 16 is the most famous and researched type of HPVs and is known as the virus to cause the cervical cancer. Even though the HD domains of both proteins have low similarity if compared to the selected peptide, PT interacted with both E2 proteins interestingly. And K_D values obtained for PT-binding E2 proteins of HPV 11 and 16 were 20.1 μM and 70.5 μM, respectively (Fig. 6c). We assume that PT interacts with the HD of HPV 11 and 16 at the motif, which are spatially close together in the three-dimensional structure. The motif is composed of amino acids, which is similar to a part of the peptide selected by phage display. The structure of HD has not been revealed, however, further structure studies are required to clarify the prediction.

In this report, we screened the peptide binding PT by T7 phage display and it was similar to a part of HD of E2 protein in HPV 1a. We also demonstrated that PT bound with HD region in E2. Thus, PT inhibits the E2/E7 interaction by binding to HD of E2 protein. These results suggest that PT has a potency to develop a seed for novel derivatives to investigate the molecular mechanism of HPV life cycle with chemical genetics approach. Structural optimization of PT as a specific inhibitor of the E2/E7 interaction and further biological effects of PT are currently underway.

4. Experimental

4.1. Compounds

Podophyllotoxin and etoposide were obtained from Sigma–Aldrich (St. Louis, MO).

4.2. T7 phage library

The preparations of T7 phage library by cDNA from *Drosophila melanogaster* and synthetic random DNAs were followed by a previous report.[20]

4.3. Synthesis of biotinylated podophyllotoxin

To a solution of podophyllotoxin (10.8 mg, 26.0 μmol) and 6-biotinylaminocaproic acid (9.6 mg, 26.9 μmol) in CH_2Cl_2-DMF (4:1, 2.5 mL) was added EDCI (14.5 mg, 75.6 μmol) and DMAP (10.9 mg, 89.2 μmol) and the mixture was stirred at room temperature for 2 days. Solvents were removed under a reduced pressure. The residue was purified on a silica gel chromatography ($CHCl_3$/MeOH = 9: 1) to yield podophyllotoxin–biotin conjugate (4.5 mg, 23%) and recovered podophyllotoxin (7.6 mg, 70%). $[\alpha]_D^{25}$ −66 (*c* 0.20, MeOH); ^{1}H NMR (600 MHz, CD_3OD) δ 6.82 (1H, s), 6.52 (1H, s), 6.43 (2H, s), 5.97 (1H, s), 5.95 (1H, s), 5.91 (1H, d, J = 13.6 Hz), 4.60 (1H, d, J = 4.6 Hz), 4.46 (1H, dd, J = 7.7 Hz, 5.0 Hz), 4.36 (1H, m), 4.28 (1H, dd, J = 7.7 Hz, 4.5 Hz), 4.22 (1H, t, J = 9.8 Hz), 3.72 (9H, s), 3.34 (1H, s), 3.19 (3H, m), 2.89 (1H, dd, J = 12.7 Hz, 5.0 Hz), 2.80 (1H, m), 2.67 (1H, d, J = 12.7 Hz), 2.48 (2H, m), 2.17 (2H, t, J = 7.3 Hz), 1.70 (2H, m), 1.61 (2H, m), 1.53 (2H, m), 1.40 (4H, m). ^{13}C NMR (100 MHz, $CDCl_3$–CD_3OD) δ 174.1, 173.8, 173.2, 163.8, 152.5, 152.5, 148.0, 147.5, 137.0, 134.8, 132.2, 128.2, 109.6, 108.0, 108.0, 106.9, 101.5, 73.4, 71.3, 61.7, 60.6, 59.9, 56.1, 56.1, 55.2, 45.5, 43.6, 40.3, 39.0, 38.7, 35.7, 34.0, 29.1, 28.1, 27.9, 26.3, 25.3, 24.4. IR (film) cm^{-1} 3288, 2934, 1775, 1699, 1650, 1589, 1542, 1505, 1484, 1460, 1421, 1376, 1332, 1239, 1158, 1126, 1037, 998, 931, 866. HRMS (ESI) calcd for $C_{38}H_{47}N_3O_{11}SNa$ ([M+Na]$^+$) 776.2823; found: 776.2841.

4.4. Cell line and cytotoxicity assay

The murine lymphocytic leukemia L1210 cell line was supported by Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University. Cells were maintained in DMEM supplemented with 10% horse serum and grown in this

medium at 37 °C in 5% CO_2/95% air. Cells were suspended at 1.0×10^4 cells in 100 μL of the media and cultured 24 h as preincubation. After cells were exposed to graded concentration of the compounds for 48 h, Cell Count Reagents SF, WST-8 (Nacalai tesque, Kyoto, Japan), was added 10 μL and cells were incubated for 4 h.[21] Then, absorbance at 450 nm was measured. Media and supplement were from Nacalai tesque (Kyoto, Japan).

4.5. Biopanning and sequence analysis

A biotinylated derivative of PT was immobilized on a streptavidin-coated 96-well plate (Nalge Nunc International, Wiesbaden, Germany) overnight at 4 °C, and unbound PT was removed by washing three times with 200 μL of 100 mM Tris–HCl (pH 8.0). Then, immobilized wells were blocked with 200 μL of Tris buffer containing 3% skimmed milk for 1 h, and washing three times with Tris buffer. Phage library solution contain 1% skimmed milk was added in well and incubated at rt for 3 h. After washing 10 times with Tris buffer in order to unbound phage particles, wells washed another ten times to eliminate non-specific bound phage particles with washing buffer (3% Tween 20, 2 M urea, 3 M NaCl, 100 mM Tris–HCl, pH 8.0). The remaining phage particles were recovered by infection to 200 μL of *E. coli* BLT5615 (Novagen, Darmstadt, Germany), were grown a log phase, and were amplified at 37 °C until *E. coli* became bacteriolysis. Lysis solutions were used in next biopanning round and picking a single plaqueup. After four rounds of this method, 96 single plaques were picked up at random from LB with 50 μg/mL carbenicillin plates and each was dissolved in a phage extraction solution (20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 6 mM $MgSO_4$). Phage DNA inserted random cDNAs was amplified by PCR using forward primer (5′-GCTAACTTCCAAGCGGACC-3′) and reverse primer (5′-TTGCCCAGAACTCCCCAA-3′, T7 select Down, Novagen). The PCR products were purified with ExoSAP-It (GE healthcare Bio-Science Corp, Piscataway, NJ) and used cycle sequence PCR with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems, Foster City, CA), then fragments were precipitated with 100% ethanol. The purified PCR products were sequenced on an ABI PRISM® 3100 Genetic Analyzer (Applied BioSystems, Foster City, CA). From results, the amino acid sequence on T7 phage capsid was determined. A homology search (BLAST) demonstrated that the amino acid sequence obtained from the selected clone, SVPSRRRPDGRTHRSSRG, is similar to that of HPV 1a hinge domain.

4.6. Affinity for PT by a selected phage single clone

The affinity of a selected clone was compared to that of a non-displayed clone which was inserted with only stop codon. The non-displayed clone was merely selected by biopanning. Both titers each clones were adjusted to 5.0×10^{10} pfu/mL. Then, 200 μL of each phage suspicion (i.e., 10^9 pfu) was incubated with Bio-PT immobilized on a streptavidin-coated 96-well plate. After washing by a same manner as above described, binding phage particles were eluted with 200 μL of Tris buffer including 1% SDS. The titers of the eluted phage particles were determined by a method described in an previous paper.[23]

4.7. Plasmid constructs

An expression vector pDF-28a (+), which has a FLAG tag, a 6 × His tag, and a multicloning site of pET-28a (+), was constructed by a method based on pET DUET-1 (Novagen). A FLAG tag fragment was generated with cloning ligated complementary synthetic oligonucleotides: 5′-TCCATGGGCAGCCATCATCATCATCATCACAGCAGCGACTACAAG-3′ and 5′-AGCCATATGTTTGTCATCGTCGTCCTTGTAGTCGCTGCTGTGATG-3′ into the NcoI/NdeI sites of pET DUET-1 instead of thrombin recognized site, and then a multicloning site of pET-28a (+) cloned with NdeI/XhoI was ligated.

HPV genomes, human papillomavirus type 1a (VG008), human papillomavirus type 11 (VG012), and human papillomavirus type 16 (VG013) were provided from the Japan Health Sciences Foundation (HSRRB, Osaka, Japan). E2 and E7 proteins of HPV in full length were derived by PCR with primers conjugated with NdeI/XhoI or NheI/XhoI restriction sites referred to National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) (1a E2 forward primer: 5′-CATATGAACCTATATGAACAGGA-3′, 1a E2 reverse primer: 5′-CTCGAGTTACAATAAATGTAATGA-3′, 11 E2 forward primer: 5′-CATATGAACCTATATGAACAGGAC-3′, 11 E2 reverse primer: 5′-CTCGAGTTAAGACCCATTAAACTG-3′, 16 E2 forward primer: 5′-GCTAGCATGGAGACTCTTTGCCAA-3′, 16 E2 reverse primer: 5′-CTCGAGTCATATAGACATAAATCCAG-3′, 1a E7 forward primer: 5′-CATATGGTGGGCGAAATGCCA-3′, 1a E7 reverse primer: 5′-CTCGAGTTACTGTCGCTGTAGGGTGC-3′), and were cloned into pDF-28a (+).

Three domains of HPV 1a E2 were constructed by PCR referred to Pfam of European Molecular Biology Laboratory (EMBL) with primers conjugating with NdeI/XhoI (1a E2 AD 1–188 forward primer to be same as full length forward primer, reverse primer: 5′-CTCGAGTTAGCTAGTGGAAGACATAAC-3′, HD 189-303 forward primer: 5′-CATATGTCCCCAAGGGCTGCTGGG-3′, reverse primer: 5′-CTCGAGTTATGGATCCCAAGCTTCCTGCA-3′, DBD 304–387 forward primer: 5′-CATATGCCCGTGGTCTGTGTAAAA-3′, reverse primer to be same as full length reverse primer), and were cloned into pDF-28a (+).

4.8. Expression and purification of recombinant HPV proteins

Protein expression was performed by transforming the constructs into Rosetta (DE3) pLysS (Novagen). These bacteria were grown up in 1 L of LB medium containing 50 μg/mL carbenicillin and 100 μg/mL

chloramphenicol. Cells were grown at 37 °C until OD_{600} came up to 0.5, and then treated 1 mM of isopropyl thio-β-D-galactoside (IPTG). After 3 h, cells were harvested by centrifugation at 3000*g* for 30 min, and were suspended up to 50 mL with the buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 20 mM imidazole, 2 M urea, 5% glycerol, 0.05% Triton X-100) and sonicated. The cell lysates were centrifuged at 20,000*g* for 10 min and the proteins were purified with 5 mL His-Trap HP column (GE healthcare) of the FPLC system (ÄKTA explorer, GE healthcare) using the elution buffer (20 mM sodium phosphate, pH 7.4, 0.5 M NaCl, 500 mM imidazole, 5% glycerol, 0.05% Triton X-100). The eluted fraction was used to confirm the purity by SDS–polyacrylamide gel electrophoresis. The buffers of eluted proteins were exchanged to the storage buffer (PBS, pH7.4, 5% glycerol) with PD-10 columns (GE healthcare) and stored at −80 °C.

4.9. Surface plasmon resonance (SPR) analysis

Proteins were immobilized on CM5 chips using an amine coupling kit (GE Healthcare) and a Biacore 3000 apparatus (GE Healthcare). The surface of chips was activated by injecting a solution containing 0.2 M *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 50 mM *N*-hydroxysuccinimide (NHS) for 10 min. Proteins diluted with 10 mM sodium acetate buffer at pH 4.0–6.0 were injected and the surface was then blocked by injecting 1 M ethanolamine at pH 8.5 for 10 min. PT at various concentration in running buffer (PBS, pH 7.4, 0.005% Tween20, 5% glycerol, 5% DMSO) were flowed at 20–30 μL/min. Regeneration of the sensor chip was used 25 mM NaOH and 0.5 mM NaCl and replicates of each experiments were performed over two times. Response curves of dissociation constants (K_D) analysis were generated by subtraction of the back ground signal generated simultaneously on the control flow cell. BIAEVALUATION 4.1 software (GE Healthcare) was used to determine the kinetic parameters.

At the observation of inhibition of E2/E7 protein–protein interaction, the flow cell of E7 immobilized was used in order to determine the amount of binding E2 protein. To avoid mass transport limitation, the flow rate was 30 μL/min. The E2 protein solution containing with DMSO, PT or EP was set just before observation.

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